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Age-Related Osteoporosis in Biglycan-Deficient Mice Is Related to Defects in Bone Marrow Stromal Cells

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ABSTRACT

Biglycan (*bgn*) is an extracellular matrix proteoglycan that is enriched in bone and other skeletal connective tissues. Previously, we generated *bgn*-deficient mice and showed that they developed age-dependent osteopenia. To identify the cellular events that might contribute to this progressive osteoporosis, we measured the number of osteogenic precursors in the bone marrow of normal and mutant mice. The number of colonies, indicative of the colony-forming unit potential of fibroblasts (CFU-F), gradually decreased with age. By 24 weeks of age, colony formation in the *bgn* knockout (KO) mice was significantly more reduced than that in the wild type (*wt*) mice. This age-related reduction was consistent with the extensive osteopenia previously shown by X-ray analysis and histological examination of 24-week-old *bgn* KO mice. Because *bgn* has been shown previously to bind and regulate transforming growth factor β (TGF-β) activity, we also asked whether this growth factor would affect colony formation. TGF-β treatment significantly increased the size of the *wt* colonies. In contrast, TGF-β did not significantly influence the size of the *bgn* colonies. An increase in apoptosis in *bgn*-deficient bone marrow stromal cells (BMSCs) was observed also. The combination of decreased proliferation and increased apoptosis, if it occurred in vivo, would lead to a deficiency in the generation of mature osteoblasts and would be sufficient to account for the osteopenia developed in the *bgn* KO mice. The *bgn* KO mice also were defective in the synthesis of type I collagen messenger RNA (mRNA) and protein. This result supports the suggestion that the composition of the extracellular matrix may be regulated by specific matrix components including *bgn*. (J Bone Miner Res 2002;17:331–340)

Key words: biglycan, osteoporosis, bone marrow stromal cells, colony-forming unit fibroblastic, collagen

INTRODUCTION

BIGLYCAN (*bgn*) is a member of the family of small leucine-rich proteoglycans known as SLRPs.[1,2] It consists of a 45-kDa core protein made almost entirely of leucine-rich repeats that are ~25 amino acids long. The *bgn* has two glycosaminoglycan (GAG) chains attached at its amino terminus and two cysteine clusters flanking each end of the leucine repeats that are engaged in disulfide bonds. The *bgn* and other members of the SLRP family including decorin, fibromodulin, lumican, epiphycan and keratocan are expressed differentially in extracellular matrices of different tissues in which they appear to perform unique functions, depending at least in part on their relative amounts.[1–3]

The *bgn* is widely distributed in the extracellular matrices of bone and specialized, nonskeletal connective tissues.[3] In both cartilage and bone, it is located at the cell surface.[3] It may interact with growth factors including transforming growth factor β (TGF-β) to regulate their biological activ-

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ity,[4] and by binding type I collagen, it also may influence the organization of the matrix,[5] although this latter effect is not always universally observed.[6] In vivo, studies with *bgn*-deficient (knockout [KO]) mice show that they develop a phenotype that resembles osteoporosis.[7] Specifically, *bgn* KO mice, beginning at 3 months of age, fail to achieve the normal increase in their bone mass. However, the cellular and molecular mechanisms responsible for this failure have not yet been identified.

The goal of this study was to identify cell types and the cellular pathways that might contribute to the osteopenia caused by a lack of *bgn*. We tested the hypothesis that the osteopenia phenotype in *bgn*-deficient mice was related to a defect in either the quantity or metabolic activity of bone marrow stromal cells (BMSCs). Our data using a colony forming unit fibroblastic (CFU-F) efficiency assay[8] suggested that *bgn* KO mice had a significantly diminished capacity to produce BMSCs and that this defect was age dependent. Some metabolic activities of BMSCs isolated from *bgn* KO mice also were abnormal. They had a reduced response to exogenous TGF-β, reduced collagen gene expression and collagen synthesis and relatively more apoptosis than BMSCs isolated from wild type (*wt*) animals. Taken together, our results indicated that osteopenia in *bgn* KO mice was associated with multiple in vitro defects in BMSCs any one of which could contribute to the defective in vivo phenotype.

MATERIALS AND METHODS

Animals

All experiments were performed using 6-, 12-, or 24-week-old *wt* and *bgn* KO male mice (C57B6/129) under an institutionally approved protocol for the use of animals in research (NIDCR-IRP-98–058). The genotype of the *wt* and *bgn* KO mice (C57BL6/129) was determined by a polymerase chain reaction (PCR)–based assay using the combination of a primer containing the PGK promoter sequences (5′-tggatgtggaatgtgtgcgagg-3′) of the targeted allele (GenBank accession no. AF090454 bases 1922–1901) along with a forward primer corresponding to the 5′-end of exon two (5′-caggaacattgaccatg-3′; (GenBank accession no. NM007542, bases 126–142) and a reverse primer corresponding to the 3′-end of exon two (5′-gaaaggacacatggcactgaag-3′; GenBank accession no. NM007542, reverse and compliment of bases 316–337) in a standard PCR reaction. PCR products were resolved by electrophoresis through 1.8% agarose gels, yielding bands that were 161 base pairs (bp) and 238 bp, representing the *wt* and targeted *bgn* KO alleles, respectively.

Culture medium

The medium consisted of α-modified minimum essential medium (MEM; Life Technologies, Grand Island, NY, USA), glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 μg/ml; Biofluids, Rockville, MD, USA), 2-mercaptoethanol (10^{-4} M), and 20% fetal bovine serum (FBS; Becton Dickinson, Franklin Lakes, NJ, USA).

Feeder cells

The femora and tibias were dissected from 4- to 6-week-old Hartley male guinea pigs and the epiphyses were removed. Bone marrow cells were flushed from the bone shafts using phosphate-buffered saline (PBS) containing 2% FBS. To prevent these feeder cells from proliferating in culture, they were γ-irradiated (caesium-137) with 6000 cGy by a Gemmacell-1000 irradiator (AECL Industrial, Kanata, Ontario, Canada).[8]

Reagents

Human TGF-β and mouse Fas-ligand were purchased from R & D Systems, Inc. (Minneapolis, MN, USA). Staurosporine (STS) was supplied by Alexis (San Diego, CA, USA). The terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) reaction staining kit for detecting apoptosis was supplied by Boehringer Mannheim (Indianapolis, IN, USA).

Colony-forming unit fibroblastic efficiency assay

The colony-forming unit fibroblastic (CFU-F) assay used was a modification of the technique originally described by Kuznetsov et al.[8] The femora and tibias of three to four *wt* or *bgn* KO mice were removed surgically and marrow cells were flushed from the bone shafts. The released cells were plated into three to five 25-cm^2 plastic culture flasks in 5 ml of culture medium containing 1×10^6 cells, incubated for 3 h at 37°C to allow attachment of adherent cells, and then washed twice with PBS to remove the nonadherent cells. Then, guinea pig feeder cells (1×10^7) were added immediately in 10 ml of culture medium containing, in some experiments, TGF-β (2 ng/ml). After 12–14 days of cultivation at 37°C in a humidified mixture of 5% CO_2 mixed with air, the cells were rinsed twice with PBS to remove nonadherent cells, fixed with 100% methanol for 20 minutes, and then stained with an aqueous solution of saturated methyl violet (Sigma Chemical Co., St. Louis, MO, USA) for 20 minutes. For alkaline phosphatase staining, the adherent cells were fixed with 95% ethanol for 10 minutes and stained with Sigma Fast 5-bromo-4-chloro-3 indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) alkaline phosphatase substrate (Sigma Chemical Co.) for 5 minutes. Colonies containing more than 50 cells were counted using a dissecting microscope. Because each colony is theoretically formed from only one CFU-F, we were able to estimate directly stem cell colony–forming efficiency simply by counting the number of colonies per 1×10^6 marrow cells originally plated. The CFU-F assay was repeated in five independent experiments at each time point.

The size and density of colonies was determined using an Alphalmager Computer System (Alpha Innotech Corp., San Leandro, CA, USA).[9] To determine the numbers of cells within colonies, the cells were detached from colonies by treating with collagenase (200 U/ml; Worthington Biochemical Corp., Lakewood, NJ, USA) and total numbers of cells per T-25 flask were counted using a hemocytometer.

Determination of apoptosis in BMSCs

Apoptosis was compared between *wt* and *bgn* KO mice in 6-, 12-, and 24-week-old mice by immunohistochemical staining with TUNEL reaction reagents following the manufacturer's recommendations. Briefly, bone marrow cells were pooled from long bones removed from three to five mice. A total of 2×10^6 cells were seeded into chamber slides in 1 ml of culture medium, incubated for 3 h at 37°C to allow attachment of adherent cells, and then washed twice with PBS to remove the nonadherent cells. Guinea pig feeder cells ($1–2 \times 10^6$) were overlaid onto each chamber slide in 1 ml of the culture medium, and the cultures were incubated until ~80% confluence (12–14 days). Then, the medium was replaced and the cells were treated with or without Fas-ligand (Fas-L; 1 μg/ml) or STS (10 μg/ml) for 48 h and 4 h, respectively. After these treatments, the adherent stromal cells were washed again twice with PBS and then fixed with 4% phosphate-buffered formalin, freshly prepared from paraformaldehyde, for 1 h at room temperature. Then, the slides were rinsed with PBS and incubated in permeabilization solution (0.1% Triton X-100 and 0.1% sodium citrate) for 2 minutes on ice (4°C). After rinsing twice with PBS, the slides were incubated with the TUNEL reaction mixture for 60 minutes at 37°C and then washed three times with PBS, incubated with secondary antibody conjugated with horseradish peroxidase for 30 minutes at 37°C, and subsequently incubated with diaminobenzidine for 10 minutes at room temperature. Negative controls were generated by staining cells previously incubated with only the secondary antibody conjugated with horseradish peroxidase.

Quantitation of apoptotic cells in culture was estimated also using trypan blue staining.[10] After 2 weeks of coculture as described previously, the cells were rinsed twice with PBS to remove the feeder cells, incubated with fresh culture medium, and treated with or without Fas-L (1 μg/ml) or STS (10 μg/ml) for 48 h and 4 h, respectively. After these treatments, nonadherent cells collected from the supernatant were pooled with the adherent cells released from the culture dish following with collagenase treatment (200 U/ml) for 15 minutes. The pooled cells were then centrifuged and resuspended in 5% trypan blue diluted with PBS. Cells were scored as apoptotic using a hemocytometer only if they showed both nuclear and cytoplasmic staining and calculated as the percentage of positive cells in the total number of cells counted.

RNA extraction and Northern blot analysis

Marrow stromal cells (10^7) harvested from long bones were seeded into 100-mm culture dishes in 5 ml of culture medium, incubated for 3 h at 37°C to allow attachment of adherent cells, and then rinsed twice with PBS to remove the nonadherent cells. Guinea pig feeder cells ($1–2 \times 10^7$) then were added into each dish in 10 ml of culture medium in the presence or absence of TGF-β (2 ng/ml) and cultured for 12 days at 37°C in a humidified mixture of 5% CO_2 with air.

Cells were rinsed three times with ice-cold PBS, and the total RNA was isolated using RNA STAT-60 Reagent (TEL-TEST "B." Inc., Friendswood, TX, USA) according to the manufacturer's protocol. The RNA was quantitated by measuring UV absorption at 260 nm and adjusted to 1 μg/μl with RNAse-free water.

A fragment of rat type I collagen $\alpha 1^{(1)}$ complementary DNA (cDNA)[11] was released as an *Eco*RI insert from the pBS/SK vector (Stratagene, La Jolla, CA, USA), separated by agarose gel electrophoresis, and recovered from the agarose using the QIAquick Gel Extraction Kit (Qiagen, Inc., Valencia, CA, USA). Similarly, a 2.4-kilobase (kb) fragment of mouse *bgn* (http://csdb.nidcr.nih.gov/csdb/frame reagents.htm) was released as an *Eco*RI/*Hin*dIII fragment from a Schlox vector (Novagen, Madison, WI, USA). These fragments were radiolabeled to specific activities of about 1×10^8 cpm/μg using [^{32}P]deoxycytosine triphosphate (dCTP) and a random primer DNA labeling kit (Stratagene, Cedar Creek, TX, USA).

RNA (10 μg) was denatured in formamide/formaldehyde, electrophoresed on 1.2% agarose gels in 1 × morpholinopropane sulfonic acid (MOPS) running buffer (ONCOR, Gaithersburg, MD, USA), transferred to Zeta-Probe GT genomic-tested blotting membrane (Bio-Rad, Hercules, CA, USA), cross-linked to the membrane by exposure to 1200 μJ of shortwave UV irradiation using a UV Stratalinker 1800 (Stratagene, Cedar Creek, TX, USA), prehybridized for 3 h at 37°C in 15 ml of Hybrisol I hybridization buffer (ONCOR), and then hybridized for 20 h with $1–2 \times 10^6$ cpm of labeled probe per milliliter of hybridization buffer. After hybridization, filters were washed twice in 2 × SSC/0.1% sodium dodecyl sulfate (SDS) for 20 minutes at room temperature followed by two more rinses in 0.1 × SSC/0.1% SDS for 20 minutes at 68°C. The filters were exposed overnight at −70°C. The steady-state levels of specific messenger RNA (mRNA)s were quantitated using a Phosphor Screen in conjunction with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). Ethidium bromide staining of 18S and 28S ribosomal RNA was used to assure equal loading of RNA in all samples.

Quantitation of [^{3}H]proline–labeled collagen

The incorporation of [^{3}H]proline into collagen was determined by a modified collagenase-digestible protein assay.[12] Briefly, cells were seeded into 6-well plates and cultured until confluence. [^{3}H]proline (10 μCi/ml) was added to each well in 1 ml of Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% FBS, 50 μg/ml of ascorbic acid, and 100 μg/ml of β-aminopropionitrile and incubated for 24 h at 37°C. The cell layer was rinsed three times with PBS, scraped from the plate in collagenase buffer (6.25 mM of $CaCl_2$, 31.25 mM of *N*-ethylmaleimide, 0.08 mM of Ca-acetate, and 6.25 mM of Tris, pH 7.4) and transferred into a Centricon microconcentrator (Millipore Corp., Bedford, MA, USA). The mixture was rinsed twice by centrifugation with 0.5 ml of collagenase buffer to remove unincorporated [^{3}H]proline and to assure complete exchange to the collagenase buffer. Then, a new tube reservoir was attached and samples were digested with 125 U/ml of collagenase (Worthington Biochemical Corp., Lakewood, NJ, USA) at 37°C in a solution of 0.25 ml of

TABLE 1. CFU-F EFFICIENCY ASSAY[a]

	Age					
	6 Weeks		*12 Weeks*		*24 Weeks*	
Experiments	wt	bgn *KO*	wt	bgn *KO*	wt	bgn *KO*
1	10 ± 4	6 ± 2	32 ± 4	28 ± 5	7 ± 1	2 ± 1
2	24 ± 3	22 ± 1	9 ± 3	10 ± 2	20 ± 3	8 ± 2
3	13 ± 3	8 ± 2	15 ± 4	12 ± 2	26 ± 4	13 ± 3
4	14 ± 3	13 ± 2	31 ± 7	15 ± 1	38 ± 2	14 ± 4
5	54 ± 3	60 ± 3	15 ± 2	13 ± 3	49 ± 7	32 ± 4
Mean ± SE[b]	22 ± 1	21 ± 1	21 ± 1	16 ± 1[c]	29 ± 1	14 ± 1[d]

[a] Number of adherent colonies per 10^6 cells plated, presented as mean ± SD from three to six flasks for each experiment.
[b] Mean ± SE computed from five individual experiments using a mixed model ANOVA.
[c] Tested by a mixed model ANOVA ($p = 0.0001$) considered highly significant in *bgn* KO compared with *wt* mice at 12 weeks of age.
[d] Tested by a mixed model ANOVA ($p < 0.0001$) considered highly significant in *bgn* KO compared with *wt* mice at 24 weeks of age.

collagenase buffer. After 3 h, the reaction was quenched by the addition of an equal volume of formamide buffer (50% formamide, 20 mM KH_2PO_4, 0.1 M KCl, pH 7.0) and the microcentricon was centrifuged at 1200*g* for 45 minutes. The remaining digested material was transferred to the bottom reservoir by two additional rinses and centrifugations with 0.5 ml of formamide buffer. The amount of collagenase-digestible material was determined by liquid scintillation counting of 1.5-ml aliquots from each reservoir. Separate aliquots of the cell pellets were used to determine DNA content based on its absorption at 260 Å. Final values were expressed as the quantity of [^{3}H]proline-incorporated–digestible protein/DNA content.

Statistical analysis

Each data point was presented as the mean ± SD calculated from three to five samples, depending on the experiments. Table 1 shows the number of CFU-F for each sample computed using a mixed model analysis of variance (ANOVA), in which age (6, 12, and 24 weeks) and group (*wt* and *bgn* KO) were fixed effects, and experiments with a nested random effect within age category. The statistical model included all main effects together with the age by group interaction effect. Adjusted estimates of group means were obtained from the model. Additionally, two sources of random variation among these counts were estimated: one among experiments and one among samples within experiments.

The other data were analyzed by an independent *t*-test. Values of $p < 0.05$ were considered as significant.

RESULTS

Comparison of the number of CFU-F (BMSCs) in bgn *KO and* wt *mice*

The number of CFU-F was determined using a CFU-F efficiency assay using "low-density" cultures. This was done so that the relative quantity of adherent stromal cells could be assessed. The relative number of colonies generated by *wt* and *bgn* KO was not significantly different from precursor cells isolated from 6-week-old mice. However, colony-forming ability of precursor cells from 24-week-old *bgn* KO mice was reduced substantially, and the appearance of colonies stained with methyl violet is shown in Fig. 1A. To more specifically identify whether the diminished colonies were related to the osteoblastic lineage, the cells were stained for alkaline phosphatase activity shown in Fig. 1B. The number of positive colonies was nearly identical to the number of colonies stained with methyl violet. Microscopic analysis showed that the positively stained cells were located in the central area of the colony, suggesting that the individual colonies may contain cells at different stages of maturation. This observation also implied that the reduction of number of colonies in *bgn* KO mice was associated with a reduction in osteoblast lineage cells. Table 1 shows the colony-forming efficiencies obtained from five independent experiments with 6-, 12-, and 24-week-old mice, and the statistical significance was determined using a mixed model ANOVA. The colony-forming ability of BMSCs isolated from 6-week-old *bgn* KO mice was not statistically different from the *wt* controls (21 ± 1 vs. 22 ± 1; $p = 0.2646$). However, the number of colony-forming cells isolated from *bgn* KO mice gradually decreased with age. The adjusted difference in number of colonies between *wt* and *bgn* KO was modest, 5 colonies (21 vs. 16; $p = 0.0001$) at 12 weeks, and large, 15 colonies (29 vs. 14; $p < 0.0001$) at 24 weeks. The reasons for the large interexperiment variation have not been identified but could, in part, be caused by differences in the feeder cells. For example, small differences in guinea pig age could result in significantly different levels of uncharacterized factors essential for colony formation. We also can not exclude the possibility that there is interanimal variability because the studies were performed using mice that are a blend of C57B6/129. However, considering the number of times the experiment was performed, we feel

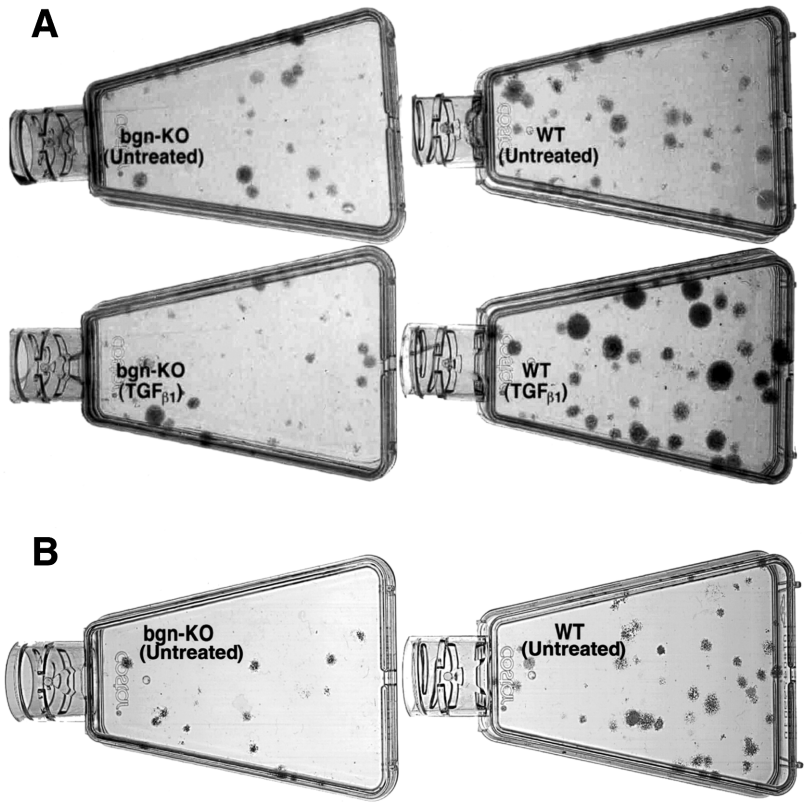

FIG. 1. The appearance of stromal cell colonies (CFU-F) derived from *wt* and *bgn* KO 24-week-old mice. Bone marrow cells harvested from femora and tibias were cultivated, processed, and stained with methyl violet as described in the Materials and Methods section. (A) Colonies stained with methyl violet. (B) Colonies stained with alkaline phosphatase.

confident that there was a statistically significant age-dependent loss in colony-forming ability in BMSCs from *bgn* KO mice.

Effect of TGF-β on marrow stromal cells from bgn *KO mice*

In an effort to identify the causes of the age-dependent loss in colony-forming capacity, we compared the effect of growth factor TGF-β on colony formation by BMSCs isolated from *wt* and *bgn* KO of 24-week-old mice. As in the previous experiment, low-density cultures were used to determine relative colony formation levels. This growth factor was chosen because *bgn* binds and regulates TGF-β activity.[4] In the presence of TGF-β, the size and staining intensity of both *wt* and *bgn* KO colonies increased, but increased more dramatically in *wt* colonies (Fig. 1A). To estimate the number of cells per colony, we used an AlphaImager, which expresses colony staining as the average number of pixels per colony (Fig. 2A). In the absence of TGF-β, the average colony staining intensity was constitutively higher in *wt* compared with the *bgn* KO mice (1009 ± 137 vs. 511 ± 218; $p < 0.05$). In the presence of TGF-β, although the number of colonies was not significantly changed (data not shown), the intensity per *wt* colony was significantly increased to 2242 ± 199 ($p < 0.05$). In contrast, the intensity per *bgn* KO colony (914 ± 242) was not statistically different from the untreated colonies (511 ± 218; $p > 0.05$). Therefore, exogenous TGF-β further increased the difference in the density per colony between the *wt* and the *bgn* KO mice. To eliminate the possibility that the increased average number of pixels per colony was caused by added matrix deposition rather than by increasing the number of cells, the cells were incubated in either the presence or the absence of TGF-β and then detached from the surface by treatment with collagenase and counted. This analysis revealed that TGF-β treatment did indeed significantly increase the number of cells from *wt* ($6.5 \pm 0.7 \times 10^5$ vs. $14 \pm 1.3 \times 10^5$; $p < 0.05$) but not from *bgn* KO mice ($4.3 \pm 0.5 \times 10^5$ vs. $5.2 \pm 0.6 \times 10^5$; $p > 0.05$; (Fig. 2B). These findings indicated that marrow stromal cells from *bgn* KO mice responded poorly, if at all, to TGF-β.

Apoptosis in marrow stromal cells from bgn *KO mice*

Because the relative number of CFU-F was significantly lower in the 24-week-old *bgn* KO mice, we wondered if BMSCs from *bgn* KO mice may be more apoptotic than *wt* marrow stromal cells. We examined this possibility by looking for apoptotic cells in cultures of 24-week-old BMSCs on chamber slides by immunohistochemistry staining with TUNEL reaction reagents. In this experiment, multicolony-derived cells were used. Apoptotic cells show darkly stained condensed round bodies as in Fig. 3. This analysis showed that stromal cell cultures derived from *bgn* KO mice had many more apoptotic cells than those derived from *wt* mice (Figs. 3A and 3B). No differences were observed in *bgn* KO and *wt* cells isolated from 6-week-old animals (data shown in Fig. 4). To understand the possible mechanisms involved in these processes, BMSCs were

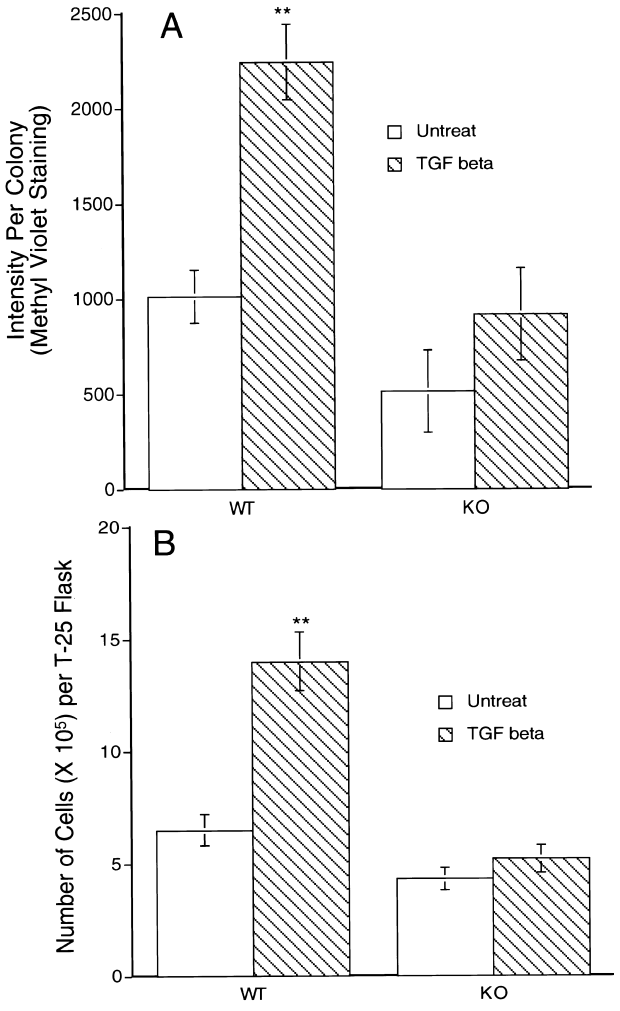

FIG. 2. Comparison of the density of CFU-F colony in *wt* and *bgn* KO 24-week-old mice in the presence or absence of TGF-β. (A) Methyl violet staining. The density per colony was calculated by measuring the total density and dividing this value by total numbers of colonies in each flask. The final values represent the mean ± SD from five individual experiments. In *wt* mice, TGF-β significantly increased the density per colony compared with the untreated mice (**$p < 0.05$). In *bgn* KO mice, TGF-β did not significantly increase the density per colony ($p > 0.05$). (B) Cell number. The cells from colonies were detached by collagenase treatment and counted. The total numbers of cells per T-25 flask were derived from four individual experiments and represented the mean ± SD. The difference between the treated and untreated mice was significant in *wt* mice (**$p < 0.05$) but not in *bgn* KO mice ($p > 0.05$).

treated further with Fas-L, STS, or TGF-β. Surprisingly, we found that cultured BMSCs from *bgn* KO mice could not be induced to undergo additional levels of apoptosis by treatment of Fas-L as easily as cultured *wt* cells (data shown in Fig. 4). In contrast, the cultured BMSCs from *bgn* KO mice were more sensitive to STS treatment than those from *wt* mice (Figs. 3C and 3D). This differential sensitivity to different apoptotic inducing agents suggests that the increased levels of apoptosis observed in the mutant cells may be associated with mitochondrial-regulated cell death rather than through the death receptor pathway. It is not possible to present reliable quantitative data on these differences because large numbers of apoptotic cells detached from the slides during the TUNEL staining procedure.

To more accurately quantitate the total numbers of apoptotic cells in cultures to compare the younger animals with the older animals (6-week-old animals vs. 24-week-old animals), the nonadherent fraction (containing substantial numbers of apoptotic cells) was pooled with adherent cells (released from the slides by collagenase treatment) and stained with trypan blue and counted using a hemocytometer. In the 24-week-old mice, the percentage of trypan blue–positive cells in untreated cultures of *bgn*-deficient cells (about 20%) was four to five times greater than in the *wt* cultures ($p < 0.05$; Fig. 4). After treatment with Fas-L, trypan blue–positive cells were significantly increased in *wt* cultures ($p < 0.05$; Fig. 4) but not in *bgn* KO cells. On the other hand, STS increased trypan blue–positive in cells from both *wt* and *bgn* KO mice; however, the percent of positive cells was significantly higher in the *bgn* KO mice than *wt* mice ($p < 0.05$; Fig. 4). In contrast, in 6-week-old mice, the trypan blue–positive cells in untreated cultures were low in both *wt* and *bgn* KO mice and increased in parallel after treatment with Fas-L and STS, in which there was no significant difference between *wt* and *bgn* KO mice (Fig. 4). TGF-β had no influence on apoptosis in BMSCs from either *wt* or *bgn* KO mice examined at all three ages (data not shown). These results confirmed the qualitative results obtained with TUNEL staining.

Comparison of type I collagen synthesis in BMSCs from wt *and* bgn *KO mice*

Our data thus far indicate that the number of colony-forming marrow stromal cells that can be isolated from 24-week-old *bgn* KO mice was significantly lower than that from *wt* mice. We next tested the biological activity of these cultured cells by looking for their ability to synthesize type I collagen mRNA and protein. Stromal cells harvested from *wt* and *bgn* KO 24-week-old mice were cultured until confluent (12 days) in the presence or absence of TGF-β. In this and the following biosynthetic experiment, multicolony or "dense" cultures were used so that all stroma cell types would be represented. Total mRNA was isolated and mRNA encoding type I collagen was determined by Northern blot analysis using a probe encoding the rat type I collagen αI cDNA.[11] Two mRNA species, ~5 kb and ~7 kb in length, were detected, consistent with mRNA sizes noted for this gene in previously published reports[11] (Fig. 5A). Interestingly, at both time points examined, the *bgn* KO cells had substantially less of both of these mRNA species than the *wt* controls. Although TGF-β increased type I collagen mRNA levels in both *wt* and *bgn* KO cells, type I collagen was always less in the *bgn* KO cells (Fig. 5A).

To determine if the reduced levels of mRNA resulted in reduced protein levels, we measured collagen production in multicolony or dense cultures derived from 24-week-old mice by radiolabeling protein with [^{3}H]proline and determining the amount of collagen synthesized by using a

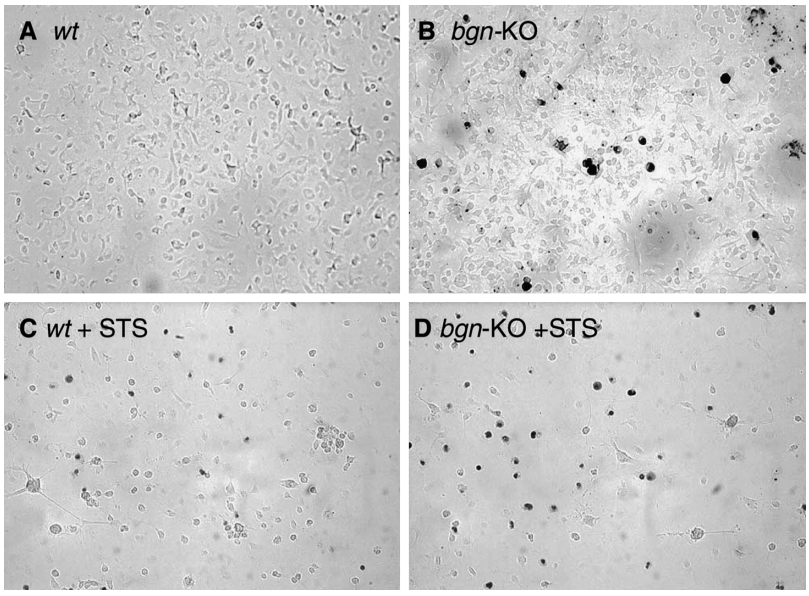

FIG. 3. Immunohistological staining for apoptosis using a TUNEL assay. Marrow stromal cells from 24-week-old mice were cultured on chamber slides and stained with TUNEL reagents. Apoptotic cells were identified by the presence of darkly stained condensed round bodies. (A) Untreated cells from *wt* mice (original magnification ×200). (B) Untreated cells from *bgn* KO mice (original magnification ×200). (C) The *wt* cells treated with STS for 4 h (original magnification ×100). (D) *Bgn* KO cells treated with STS for 4 h (original magnification ×100).

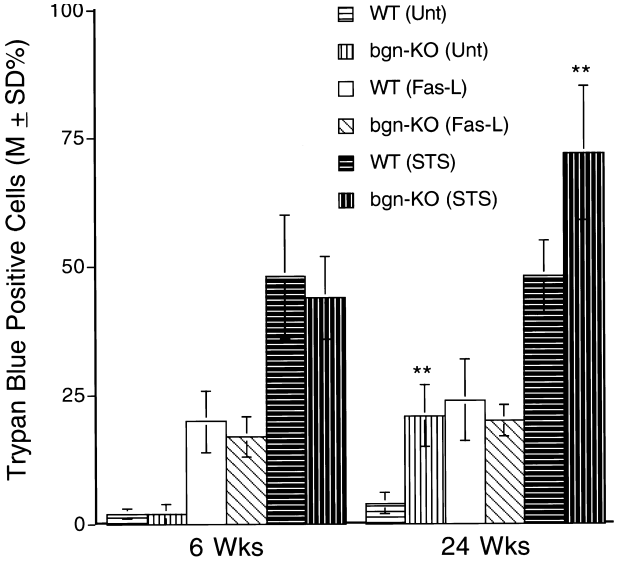

FIG. 4. Quantitation of apoptotic cells using trypan blue staining. Marrow stromal cells from either 6-week-old or 24-week-old mice were cultured in the presence or absence of Fas-L or STS, as described in the Materials and Methods section. In 24-week-old mice, *bgn* KO cells contained significantly more positive cells than those from *wt* when either untreated (Unt) or treated with STS (**$p < 0.05$). In contrast, both *wt* and *bgn* KO cells from 6-week-old mice had the same low number of positive cells, and the difference between *wt* and *bgn* KO was not induced by treatment with Fas-L or STS.

collagenase digestion assay.[12] When the amount of collagenase-digestible [^{3}H]proline was measured and normalized to cell number (DNA content), it was found that stromal cells derived from *bgn* KO mice synthesized approximately one-half as much collagen as the *wt* mice (Fig. 5B). Considering that multicolony-derived cells were used for this experiment, it could be that different cell populations could be responding differently, which, in turn, could mask or confound our interpretations. Nevertheless, taken together, these findings suggested that the uninduced and TGF-β–induced levels of collagen mRNA and protein in cells from *bgn* KO mice were compromised.

DISCUSSION

Previous studies have shown that mice rendered deficient in *bgn* (*bgn* KO) developed age-dependent osteopenia having a phenotype resembling osteoporosis.[7] Although double-labeling experiments showed decreased bone formation in *bgn* KO mice, the cell and molecular mechanisms for this defect were not identified. To increase our understanding of this phenotype, we examined both the quantity and the metabolic activity of bone precursor cells derived from the BMSC population.

BMSCs are multipotential stem cells that give rise to osteoblasts, chondrocytes, adipocytes, fibroblasts, and perhaps even muscle cells.[13] In this study, we asked if this age-dependent osteopenia could be caused by a decrease in the number of biologically competent osteoblast precursors. Our experimental approach was indirect; we isolated and cultivated bone marrow osteoblast precursor cells and examined them in a number of different assays. When we evaluated the numbers of progenitors (CFU-F) using a CFU-F efficiency assay, we showed that the ability to form colonies was significantly reduced in cells derived from 24-week-old *bgn* KO mice. Moreover, there appeared to be a trend in the pattern of number of colonies in which the difference between the *wt* and *bgn* KO mice increased with age. Interestingly, alkaline phosphatase positive colonies also were diminished at 24 weeks in the *bgn* KO cells, implying that there was a parallel decrease in available CFU-osteoblastic (OB). This observation by itself might be sufficient to explain the pronounced osteopenia shown by

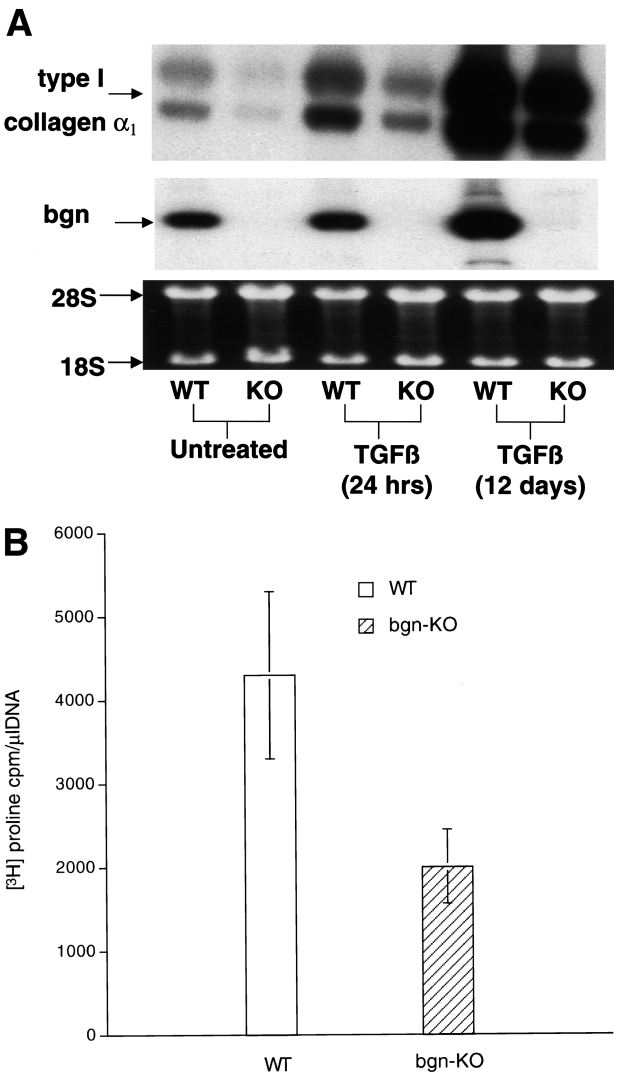

FIG. 5. Type I collagen mRNA and collagen synthesis in marrow stromal cells derived from 24-week-old *wt* and *bgn* KO mice. (A) Northern blot analysis. Total RNA was separated by electrophoresis, transferred to nylon membrane, and probed with a radiolabeled rat type I collagen α1 (I) or a *bgn*-specific probe. The ethidium bromide staining of 18S and 28S ribosomal RNA was used to assure equal loading of RNA in all samples. (B) Quantitation of [^{3}H]proline-labeled protein digestible with collagenase. The values shown are normalized to the DNA content in each sample. Values are the mean ± SD from four experiments.

X-ray analysis and histological examination of long bones isolated from these mice.[7] We then considered two possible causes for the apparently reduced population of potential osteoblast precursor cells: reduced response to growth factors and increased apoptosis.

Because *bgn* can bind and potentially regulate TGF-β activity,[4] we theorized that the lack of *bgn* might interfere with the normal effects of TGF-β on BMSCs function. To test this hypothesis, we examined the ability of *bgn*-deficient BMSCs and their descendants to respond to TGF-β as measured by colony-forming efficiency and type I collagen production, respectively. We found that TGF-β was not able to increase the number of either *bgn* KO or *wt* colonies. However, TGF-β significantly increased both the size and the density of cells in the colonies in *wt* mice compared with colonies derived from the *bgn* KO mice. It should be noted that this assay has limitations: it measures only cells that can attach and proliferate under the conditions used. We will consider two possibilities for why *wt* but not *bgn* KO BMSCs formed larger colonies in response to TGF-β. First, the *bgn*-deficient BMSCs may have limited capacity to divide. This possibility is consistent with our observation that as the *bgn* KO mice become older, they fail to produce a normal number of osteoblast precursor cells. A second possibility is that *bgn*-deficient BMSCs have or develop decreased sensitivity to TGF-β. We speculate that *bgn* and other extracellular matrix proteins such as decorin (dcn) may competitively bind TGF-β and have opposite effects on TGF-β activity. Some studies suggest that dcn inhibits TGF-β activity by direct binding.[4] Clearly, additional experiments are required to understand why *bgn*-deficient stromal cells respond differentially to TGF-β.

A second possible cause for the reduced level of BMSCs detected in the CFU-F assay of the 24-week-old *bgn* KO mice is an increased amount of BMSC apoptosis. As predicted, increased apoptosis was detected in cultures of these cells with both TUNEL and trypan blue exclusion staining assays.[14] The increased in vivo apoptosis or increased susceptibility to apoptosis during experimental manipulations could explain the decreased colony formation capacity of BMSCs isolated from the 24-week-old *bgn* KO mice (Fig. 2). The increased apoptosis also may explain the previous findings in vivo in which the number of osteoblasts is reduced in the *bgn* mutant mice.[7] It has been hypothesized that some apoptosis normally occurs and may even be necessary for the normal differentiation of osteoblasts.[10] On the other hand, too much apoptosis of osteoblasts and osteocytes such as occurs after glucocorticoid treatments can cause osteoporosis.[15,16] Bisphosphonates can inhibit glucocorticoid-induced osteoporosis by preventing the osteoblast and osteocyte apoptosis induced by the glucocorticoids.[17] In addition, parathyroid hormone appears to increase bone formation by inhibiting apoptosis of osteoblasts.[18] Taken together, these studies suggest that controlled apoptosis may normally play an important role in regulating the osteoblast life cycle or life span in physiological differentiation and that both abnormally low and high levels of apoptosis can lead to pathological conditions.

Our experiments provided some evidence about the signaling pathway involved in the enhanced apoptosis occurring in the *bgn* KO cells. Apoptosis was promoted in both *wt* and *bgn* KO cells by treatment with STS, a caspase-independent mitochondrial death effector.[19,20] On the other hand, Fas-L induced apoptosis only in *wt* BMSC cultures. The failure of Fas-L to further stimulate apoptosis in cultures from *bgn* KO mice can be caused by either lack of Fas-L receptors on the cell surface or to the absence of Fas-L receptor–expressing cells associated with mitochondrial-regulated cell death. These observations suggest that apoptosis in the *bgn* KO cells might not be elicited through the usual "death receptor" pathway (Fas/APO-1/CD95),[21] but

rather, may be associated with mitochondrial-regulated cell death. Therefore, it is conceivable that the activation of this pathway in part may be important in the age-dependent osteoporosis occurring in humankind.

We also asked if these *bgn* KO cells were defective in their ability of synthesize any other components of the extracellular matrix. We found that cells derived from the 24-week-old *bgn* KO BMSCs were unable to synthesize normal amounts of type I collagen mRNA or protein. In this regard, it is tempting to speculate that the *bgn* KO cells have an impaired differentiation capacity. Although the *bgn* KO cells were responsive to exogenous TGF-β, the induced levels were less than the *wt* controls. The decreased type I collagen production could cause abnormalities in skeletal microarchitecture and might explain why long bones isolated from 24-week-old *bgn* KO mice have compromised biomechanical strength.[7] The reduced collagen production also might explain the reduced mineral apposition rate observed in vivo in *bgn* KO bones.[7] The possibility that the composition of the matrix also influences, in a complex manner, the expression of genes encoding these matrix proteins is consistent with experiments done with cells derived from patients with osteogenesis imperfect with decreased type I collagen expression. These experiments showed a coordinate decrease in both *bgn* and dcn protein synthesis.[22] The role of the matrix and its components in osteoblast differentiation appears to be highly complex.[23] Additional experimentation will provide a better understanding of how these matrix components influence gene expression.

An important feature of the *bgn* KO phenotype is that the reduced bone acquisition is age dependent. Possible reasons why *bgn* deficiency does not cause a detectable phenotype at an earlier age are that in young animals the *bgn* function is not required or that it is redundant; for example, it could be compensated for by increased synthesis of other members of the SLRP family. Indeed, apparently redundant matrix functions are already known. KO mice deficient in fibromodulin grow to a normal size, are fertile, have a normal life span, and synthesize increased levels of the related SLRP, lumican.[24] The changing expression pattern of the human SLRPs with age also is consistent with a compensation model. Both dcn and *bgn* are synthesized at a peak rate at puberty, which then gradually decreases over time, reaching minimum levels in cells from patients over 40 years of age.[25] This observation invites the prediction that the pathological changes in the *bgn* KO mice will become progressively worse with time, simply because other SLRPs are slowly becoming less abundant and less available for compensating the *bgn* defect. Experiments with double KO mice could be used to determine the potential compensatory role of the different SLRPs in skeletal cell and tissue function.

In this report, we showed that *bgn* deficiency causes defects in the quantity and normal activity of BMSCs. *Bgn* KO mice developed less alkaline phosphatase–positive stroma cells implicating the presence of less CFU-OB, perhaps, because of increased apoptosis. The *bgn* KO BMSCs also responded weakly to TGF-β and produce less type I collagen mRNA and collagen protein. Any one of these defects alone or any combination of them could contribute to the osteopenia that develops in *bgn* KO mice. This article provided an important foundation to understand the age-dependent development of osteopenia caused by a deficiency of a single bone matrix protein.

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